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## Current Research in Insect Science

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# Investment in adult reproductive tissues is affected by larval growth conditions but not by evolution under poor larval growth conditions in *Drosophila melanogaster*☆

Rohit Kapila, Soumyadip Poddar, Neeraj Meena, Nagaraj Guru Prasad\*

Department of Biological Sciences, Indian Institute of Science Education and Research Mohali, Sector 81, SAS Nagar, PO Manauli, Mohali, Punjab 140306, India

## ARTICLE INFO

## Keywords:

Larval Crowding  
 Accessory glands  
 Testis  
 Experimental evolution  
*Drosophila melanogaster*  
 Density Dependent Selection

## ABSTRACT

In many insects, the larval environment is confined to the egg-laying site, which often leads to crowded larval conditions, exposing the developing larvae to poor resource availability and toxic metabolic wastes. Larval crowding imposes two opposing selection pressures. On one hand, due to poor nutritional resources during developmental stages, adults from the crowded larval environment have reduced investment in reproductive tissues. On the other hand, a crowded larval environment acts as a cue for future reproductive competition inducing increased investment in reproductive tissues. Both these selection pressures are likely affected by the level of crowding. The evolutionary consequence of adaptation to larval crowding environment on adult reproductive investment is bound to be a result of the interaction of these two opposing forces. In this study, we used experimentally evolved populations of *Drosophila melanogaster* adapted to larval crowding to investigate the effect of adaptation to larval crowding on investment in reproductive organs (testes and accessory glands) of males. Our results show that there is a strong effect of larval developmental environment on absolute sizes of testes and accessory glands. However, there was no effect of the developmental environment when testis size was scaled by body size. We also found that flies from crowded cultures had smaller accessory gland sizes relative to body size. Moreover, the sizes of the reproductive organs were not affected by the selection histories of the populations. This study highlights that adaptation to two extremely different developmental environments does not affect the patterns of reproductive investment. We discuss the possibility that differential investment in reproductive tissues could be influenced by the mating dynamics and/or investment in larval survival traits, rather than just the developmental environment of the populations.

## 1. Introduction

Among many other factors of the juvenile environment, two factors that can directly affect adult reproductive fitness are a) nutritional resources and b) environmental cues for future reproductive competition. Crowded larval environments are characterized by high competition for resources and the accumulation of toxic excretory waste (urea) in the environment (Botella et al., 1985). In a crowded larval culture, on one hand, poor nutritional resources will reduce the nutritional uptake of the organism leading to a smaller body size (Mirth and Shingleton, 2012) and a hence smaller absolute investment in reproductive tissues. On the other hand, an increased number of larval competitors in a crowded environment can act as a cue for increased levels of reproductive competition that an adult male will face (Bretman et al., 2016). In many holometabolous insects, the allocation of resources and the development

of various tissues that affect adult reproductive fitness happens in the pre-adult stages. Therefore, if male larvae can perceive the risk of future reproductive competition based on the larval density, and accordingly allocate resources to the development of various reproductive tissues, they are likely to have a fitness advantage (Liu et al., 2021). Therefore, the evolution of adult reproductive traits under crowded larval environments is likely to be affected by the two conflicting factors: available nutrition and competitive cues.

Multiple studies have addressed the hypothesis that larger testis size (either as an evolved increase in size or a plastic increase in size) is advantageous in the face of increased sperm competition. Larger testis size has been shown to be associated with higher levels of sperm competition at a cross-species level (Lüpold et al., 2020). In *Drosophila*, while testis size is known to evolve in response to extreme female biased operational sex ratio (Reuter et al., 2008) and enforced monogamy (Pitnick et al., 2001), both, multigenerational experimental evolution

Abbreviations: MB, Melanogaster baseline population; MCU, Melanogaster crowded as larvae uncrowded as adults.

☆ Larval crowding and reproductive investment, reproductive tissues.

\* Corresponding Author: Nagaraj Guru Prasad

E-mail address: [prasad@iisermohali.ac.in](mailto:prasad@iisermohali.ac.in) (N.G. Prasad).

<https://doi.org/10.1016/j.cris.2021.100027>

Received 26 February 2021; Received in revised form 9 December 2021; Accepted 10 December 2021

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studies (Crudgington et al., 2009; Chechi et al., 2017) and a single generation study (Bretman et al., 2016) have found that testis size does not increase with increased levels of sperm competition. Similarly in *C. maculatus*, no evidence of increase in testis size was found in males evolving in male biased populations (McNamara et al., 2016). Other studies have explored plasticity in testis size in relation to larval density. The effect of larval density on plastic investment in testis size is variable across species, with some studies showing increase in body size scaled (Gage, 1995; Stockley and Seal, 2001) and absolute size of testis (Johnson et al., 2017), and other studies showing smaller absolute testis size (Liu et al., 2021) in the males grown under higher larval density. In *D. melanogaster*, the testes size of males grown under high and low larval density are not different from each other (Bretman et al., 2016). Additionally, in *D. melanogaster* (Bangham, Chapman and Partridge, 2002) did not find a correlation between testis size and pre or post copulatory success of males. Thus, the evidence for the fitness advantage of the larger testis size (either in terms of evolved size or a plastic increase in size) in relation to increased sperm competition is equivocal and is likely to depend on a lot of factors that vary between species and contexts.

In many insect species, along with the testis, the accessory glands play a significant role in determining male fitness (Hopkins, Avila and Wolfner, 2018). The seminal proteins of males, secreted by accessory glands, can manipulate the physiology of the reproductive tract and the behavior of females in a way that confers a fitness advantage to the male in post-copulatory competitive scenarios (Baldini et al., 2012; Leopold, 1976; Wolfner, 1997). In *Drosophila*, males with larger accessory glands mate more often than males with smaller accessory glands (Bangham, Chapman and Partridge, 2002) as they can potentially have access to enough resources (possibly through a larger pool or differential rate of use or replacement of resources) to re-mate often and maintain high reproductive success for longer. In contrast, males with smaller accessory glands resources refrain from mating frequently because they can suffer a courtship cost without fertilization benefits. The positive association between the size of accessory glands and the mating rate of males is also found in other species like the stalk-eyed flies (Baker et al., 2003). While some studies have found that accessory glands evolve to be larger in response to increased male-male competition (Crudgington et al., 2009), others did not find this relationship (McNamara et al., 2016; Reuter et al., 2008; Chechi et al., 2017). Additionally, it is also known that males of *Drosophila* when grown at low larval density (hence larger body size) produce more of two key seminal proteins: sex peptide and ovulin, than males grown at high larval density; whereas, small males transfer more stored seminal proteins than the large males (Wigby et al., 2016). However, to the best of our knowledge, only one study has looked at the effects of larval crowding on the size of accessory glands, and found that *D. melanogaster* males from high larval density have larger accessory glands compared to males from low larval density (Bretman et al., 2016), presumably in anticipation of the increased reproductive competition that the adult males are likely to face.

To summarize, while there are studies suggesting a fitness advantage of bigger testis and accessory glands for males, the overall evidence for fitness benefits of bigger testis/accessory glands is equivocal. Additionally, while we know a lot about the strategies used in the adult environment (Bretman et al., 2009; Friberg, 2006; Garbaczevska et al., 2013; Maggu et al., 2021; Nandy and Prasad, 2011; Rouse and Bretman, 2016), we know less about plastic investment strategies in the developmental environment. Interestingly, since both testis and accessory glands, just like other reproductive traits, are likely to be costly and trade-off with other important life-history traits (Hosken, 2001; Scharf et al., 2013; Schwenke et al., 2016). It is possible that under resource-limiting conditions, the size of one organ may potentially trade-off with the other, leading to complex effects on male reproductive fitness.

There are two important issues to note about all the studies that have assessed the effect of larval density on adult reproductive tissue size: (a) all these are single generation studies and hence yield an understanding

of the phenotypic correlation between larval density and adult reproductive tissue size and (b) in all these studies, resources were not severely limiting and thus the effects seen are likely that of density alone (and may not include the effects of resource limitation, toxic metabolic wastes etc. that usually accompanies increased larval density). Therefore, the evolution of resource investment into reproductive tissues in males of populations that experience larval crowding every generation is not yet understood.

A recent experimental evolution study showed that adaptation to a larval crowding environment leads to the correlated evolution of increased pre-copulatory behavior in populations of *Drosophila melanogaster* (Shenoi et al., 2016). However, little is known about the evolution of investment in reproductive tissues of males as a correlated response to adaptation to the poor developmental environment in these populations.

In the present study, we use eight populations of *Drosophila melanogaster*, four of which are adapted to high-density crowded larval environments for more than 165 generations, and the other four are their low larval density controls. We addressed the following questions:

1. How does resource allocation to reproductive organs (testes and accessory glands) of males change with a change in the larval density?
2. What are the evolutionary consequences of adaptation to crowded larval environments in terms of resource-allocation to reproductive organs in males?

We have quantified the size of testes and accessory glands as a proxy for investment in reproductive tissues. In our experiment, we measured the size of the reproductive organs of males of two different ages: 4-days old and 9-days old. These specific ages were selected due to their relevance to the maintenance regime of these populations.

## 2. Materials and methods

This study involved eight laboratory populations of *Drosophila melanogaster*, four of which are selected for adaptation to larval crowding, namely: MCU (Melanogaster Crowded as larvae, Uncrowded as adults: 1-4) and the remaining four are their respective controls MB (Melanogaster Baseline controls: 1-4) which are adapted to normal (uncrowded) larval densities. These populations are the same as described in (Shenoi et al., 2016). The MB and MCU populations were originally derived in the laboratory of Professor Amitabh Joshi and were provided to us in February 2011, at which point they had undergone over 75 generations of selection. These populations were maintained in our laboratory for more than 165 generations before beginning the current set of experiments.

Each selected population (MCU 1-4) was derived from its respective ancestral MB population (i.e., MCU 1 was derived from MB 1 and so on) and maintained as a separate population. MCUs connected to MBs by the same replicate numbers are their direct descendants and are used as statistical blocks representing ancestry in analyses.

### 2.1. Population maintenance regime

All eight populations (MB 1 – 4 and MCU 1 – 4) are maintained on a 21-day discrete generation cycle on standard cornmeal-charcoal food (food recipe is detailed in supplementary materials); at 25°C (24: 0 light: dark period) and 50% humidity on a 21-day generation cycle. The populations (MCUs = Melanogaster Crowded as larvae, Uncrowded as adults; and MBs = Melanogaster Baseline, uncrowded as larvae, uncrowded as adults) were named according to the larval and adult densities at which they were maintained.

Briefly, the maintenance regime of MCUs is the same as MBs except: 1- Every generation, in MCUs, 800 eggs are collected in 1.5 ml of charcoal-cornmeal food as compared to 60 eggs in MBs in 6ml of food per vial. Because of crowding, food runs out in MCUs vials in 3-4 days (hence larval competition because of crowding). It is noteworthy that

food is not re-filled in the vials once it runs out, and larvae continue to develop in the foodless vial.

2- Every generation, 24 vials of 800 eggs are collected per population in MCUs (19,200 eggs per block) as compared to 40 vials of 60 eggs (2,400 eggs per block) in MBs. Since there is tremendous pre-adult mortality in MCU and almost zero pre-adult mortality in MB, the adult population size in both regimes is close to 2,400 every generation.

3- Because of crowding, the eclosion pattern of adults changes and spreads over 10 days, hence from day 8 onwards, eclosing adults of MCUs are transferred into a cage daily till day 18, to avoid any adult crowding. Whereas for MBs, since there is no crowding in vials, all the adults eclose by day 12 post egg collection, and hence they are transferred to cages on day 12 as described above.

## 2.2. Standardization and generation of experimental flies

To remove any non-genetic parental effects (Rose, 1984) all the populations were standardized for one generation, where they were subjected to similar relaxed conditions before using them for experiments. The 'relaxed conditions' are the conditions where there is no selection pressure of larval crowding on both the selected and control populations. For the process of standardization, eggs are collected from the selected and control populations at a density of 300 eggs per 40-50 ml of food in four different bottles. On day 10 post egg collection, when all the adults from the culture eclose, flies from all the four bottles of a population are transferred into a Plexiglas cage (24 cm x 19 cm x 14 cm) containing a Petri-plate of cornmeal-charcoal food and wet absorbent cotton for maintaining a high humidity level. Therefore, for each standardized population, we have a total of approximately 1200 flies. Egg collection for the experiments was done from the standardized flies. Thirty-six hours before experimental egg collection, a food plate with ad libitum yeast paste was given. A 6-hour egg-laying window was given to standardized flies fed with yeast for experimental egg collection.

For our experiments, both selected and control populations had two treatments:

1. The high-density (HD) treatment had 600 eggs per vial containing 2 ml of food.
2. The low-density (LD) treatment had 60 eggs per vial containing 6 ml of food.

It is noteworthy that in our assays, we have used 600 eggs per vial as the high-density treatment for both the crowding adapted (MCU) and the control (MB) populations. During usual maintenance, MCU populations are held at a density of 800 eggs per vial. Using this density was necessary because MB population larvae do not survive to adulthood if grown at 800 eggs per vial density. Therefore, in the high-density assay conditions, the MCUs were under slightly lower density compared to their normal maintenance.

Larval crowding and adaptation to crowding affect development time by increasing the variation and decreasing the mean in *D. melanogaster* populations (Santos et al., 1997). Therefore, egg collection for different populations and treatments was done on different days to ensure that all the adults were of the same age post-eclosion on the day of the experiment. By staggering the egg collection to match adult age across treatments and collecting all the flies eclosing out of HD cultures, we ensured that there is no bias in our sampling because of developmental-time-induced body size variation. Separate egg collection was done for each assay; i.e., flies for dissection of accessory glands and testes at different ages came from different sets of egg collection done specifically for that assay.

During the regular maintenance cycle, on day 18<sup>th</sup> post egg collection, when the flies are about 8-9 days old as adults, flies are provided with yeast paste supplement along with their normal charcoal-cornmeal food. The 48-hour long yeasting period is followed by an 18-hour egg collection period. Eggs laid by flies only during these 18 hours are used for the next generation. Additionally, from our previous studies (Shenoi et al., 2016) and unpublished data, we know that the yeasting

event on day 18 post egg collection marks the peak in reproductive activities in these populations. Therefore, in this study, we sampled the males from a day before the reproductive peak period (4-day old adults), and a day that falls within their reproductive peak period (9-day old adults).

## 2.3. Testis dissection

By the 9<sup>th</sup> day post egg collection, in the high larval density treatment (HD), adults start to eclose. These adults were transferred into the Plexiglas cages (12 cm x 11 cm x 11 cm) with enough food and a non-crowded environment daily. Whereas flies from low larval density (LD) treatments were transferred into the cages on day 12 post egg collection. The protocol of transferring HD flies in cages daily and transferring LD flies into cages 12-day post egg collection is a standard procedure used in larval crowding related studies to ensure that there is no crowding or resource limitation in adult stages (Nagarajan et al., 2016; Sarangi et al., 2016; Shenoi et al., 2016). The maintenance of experimental flies was done mimicking the maintenance regime of our larval crowding selected (MCU) and larval crowding control (MB) populations. Until the day of assay, flies were maintained in the cages and were provided with a fresh food plate and moist cotton every alternate day. On the day of the experiment, in separate assays, 4-day old and 9-day old adult males were randomly sampled from the cages and were transferred into food vials. Shortly after that, a virgin female from an ancestrally related population (details of the population are described in the supplementary material) was introduced into the vial, and mating was observed for an hour. Since studies have shown that the mating status of males has an impact on the size of their testis and accessory glands in *Drosophila* (Linklater et al., 2007; Hopkins et al., 2019), by observing single mating, we ensured that all the males were of the same 'non-virgin' mating status in our assays. Throughout the experiment, just like the regular maintenance regime of the stock, all the flies were kept at 25°C, 60–80% RH, 24-hour light regime. Males that mated were frozen and kept at -20°C until the dissections were done. Freezing the males does not affect the size of their reproductive tissues (Chechi et al., 2017). From the frozen males, randomly chosen 20 males per population and density treatment were dissected for every block. Before dissection, all the frozen males were taken out from -20°C to be thawed and brought to 25°C. Dissections were done under a compound microscope (Leica MC120HD, Leica Microsystems GmbH, Wetzlar, Germany) and photographed at 40 × using the attached digital camera connected with Leica Stereo Zoom Microscope (M 205C, Leica Microsystems GmbH, Wetzlar, Germany). From every dissected male, an image of the testes and the left-wing was taken. All the testes were dissected on a glass slide in 1x PBS solution, and were uncoiled completely using fine forceps before imaging.

## 2.4. Accessory gland dissection

Similar to testis dissection, in separate assays, 4-day old males and 9-day old males were randomly sampled from the cages and kept at -20°C until the dissections were done. All the dissection was done in 1x PBS, on a glass slide under a compound microscope (Leica MC120HD, Leica Microsystems GmbH, Wetzlar, Germany) and was photographed at 40x zoom using the attached digital camera connected with Leica Stereo Zoom Microscope (M 205C, Leica Microsystems GmbH, Wetzlar, Germany). Thirty males per population and density treatment were dissected for every block.

Testes in *Drosophila melanogaster* are heavily coiled structures. To measure their area, it was necessary to uncoil them completely. The process of uncoiling the testes at times could lead to damage of accessory glands of the sample. Therefore, we dissected separate flies for testis and accessory gland dissections. The samples that were improperly dissected or were not imaged properly were not used for further analysis. A final count of the samples analyzed for each treatment per block is mentioned in the supplementary material (Supplement Table S3 and Table S4).

**Table 1**

Summary of three-factor mixed model ANOVA with selection and density treatment as fixed factors crossed with random blocks on the absolute area of testis (a) 4 days old males (b) 9 days old males. Significant terms are marked with “\*” sign.

|                                       | (a)   |     |       |        |       | (b)   |     |       |        |       |
|---------------------------------------|-------|-----|-------|--------|-------|-------|-----|-------|--------|-------|
|                                       | SS    | DF  | MS    | F      | p     | SS    | DF  | MS    | F      | p     |
| Intercept                             | 42.05 | 1   | 42.05 | 522.47 | 0.01  | 24.12 | 1   | 24.12 | 1812.5 | 0.01  |
| Selection                             | 0.01  | 1   | 0.01  | 0.01   | 0.95  | 0.01  | 1   | 0.01  | 0.96   | 0.41  |
| Density treatment                     | 2.1   | 1   | 2.1   | 539.43 | 0.01* | 0.66  | 1   | 0.66  | 75.46  | 0.01* |
| Block                                 | 0.25  | 3   | 0.09  | 19.61  | 0.53  | 0.05  | 3   | 0.02  | 1.18   | 0.43  |
| Selection × Density treatment         | 0.01  | 1   | 0.01  | 0.08   | 0.81  | 0.01  | 1   | 0.01  | 0.04   | 0.86  |
| Selection × Block                     | 0.03  | 3   | 0.01  | 1.03   | 0.5   | 0.02  | 3   | 0.01  | 3.13   | 0.19  |
| Density treatment × Block             | 0.02  | 3   | 0.01  | 0.42   | 0.76  | 0.03  | 3   | 0.01  | 6.7    | 0.08  |
| Selection × Density treatment × Block | 0.03  | 3   | 0.01  | 2.97   | 0.04  | 0.01  | 3   | 0.01  | 0.92   | 0.44  |
| Error                                 | 0.77  | 243 | 0.01  | 0      | 0     | 0.31  | 213 | 0.01  | 0      | 0     |

### 2.5. Measurement of the testis, accessory gland area, and wing size

NIH Image J version 1.50b was used to measure the area of testes and accessory glands, and length of the wing. An image of a standard stage micrometer (1 mm) glass slide was taken before imaging each organ mount at the same magnification (40 ×). The micrometer image served as a reference for the organ (for absolute length/pixels). Each image was analyzed manually by outlining the tissue using the ‘Polygon selections’ tool in image J. The area enclosed was then measured using the ‘Measure’ option provided under the ‘Analyze’ section of Image J. For wing size, we used the ‘Straight line’ tool in image J to join the anterior and posterior end of the longest vein of the wing. The selected length was measured using the ‘Measure’ option provided under the ‘Analyze’ section of Image J (see Fig. S2 and Fig. S3 of the supplementary materials for stepwise images of how the measurement was done). The area of each organ was measured twice by an observer who was blind to the treatments. The average of the two measurements was taken and was used as the unit of analysis. For wing size, the length of second longitudinal vein was measured twice from the anterior crossvein to the end of the vein, and the average of both the readings was taken. For both accessory gland and testis, we calculated two types of area: 1) Absolute size, 2) Body normalized size. The values obtained from the average of two measurements of the area of organs was called the ‘Absolute size’ of that organ, whereas the area of an organ obtained by dividing the absolute size with the square of wing size for each sample was called the ‘Scaled size’ of that organ.

### 3. Statistical Analysis

Three-factor mixed-model ANOVA was used to analyze the absolute organ area and normalized organ area using selection regime and density as fixed factors crossed with block as a random factor. All analyses were done at  $\alpha = 0.05$  level of significance, using Statistica for WINDOWS, version 10. Multiple comparisons were done using Tukey’s HSD post-hoc test.

Since the experiments for different ages were done from a different set of flies on different days, the data across ages were not comparable. Therefore, in all our analyses, the data of each age were analyzed separately.

### 4. Results

We collected data separately for the two ages to see if the effect of the selection history and larval density on MCUs and MBs at 4-days old males was similar to the difference between the two populations at 9-day old males. Our results suggest that at both ages, the effect of selection history and larval density treatment was similar (Fig. 1–4) (Table 1–4).

In both the ages, there was no significant effect of selection regimes as both absolute and body size scaled area of the testis (Table 1 and 2) (Fig. 1 and 2) and accessory glands (Table 3 and 4) (Fig. 3 and 4) were similar in males of MCU and MB populations.

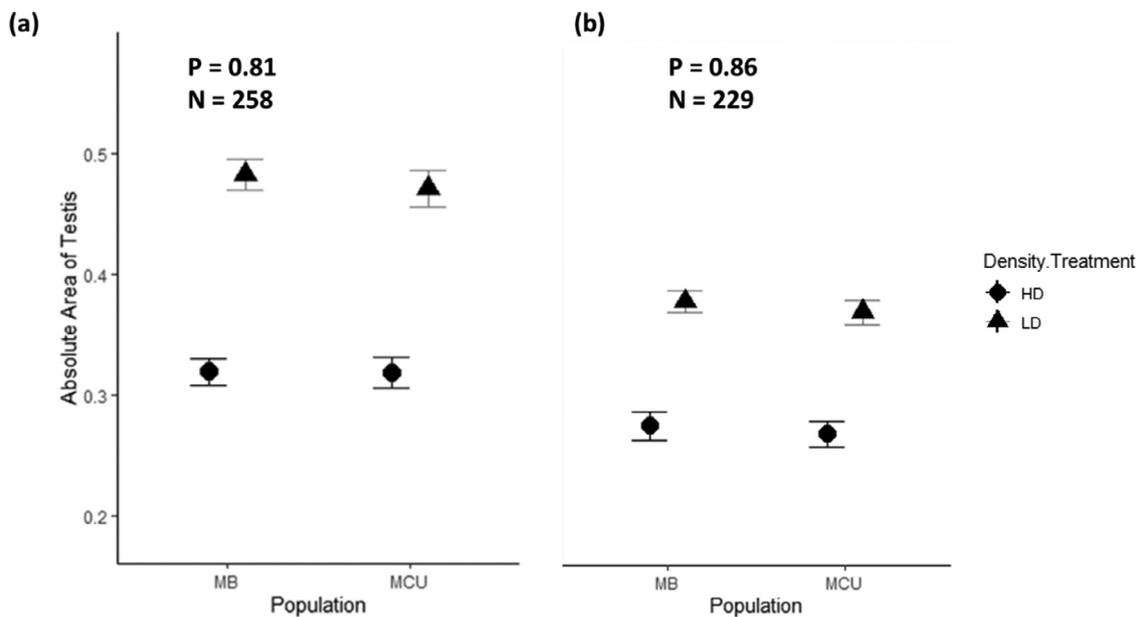
Additionally, for both the ages, we found a significant effect of larval density treatment on the absolute area of the testis (Table 1) (Fig. 1) and accessory gland size (Table 3) (Fig. 3), with males from low larval density treatment having bigger testis and accessory gland size than the males from high larval density treatment. When the absolute areas of testes and accessory glands were scaled for body size, we did not find the effect of larval density treatment on the area of the testes (Table 2) (Fig. 2). In contrast, the males of high larval density treatment had a significantly smaller body size scaled area of accessory glands than those from low larval density treatment (Table 4) (Fig. 4). This result suggests that in both selected and control populations, the proportion of investment of resources in testis does not change with change in a larval growth environment, whereas the proportion of investment of resources in accessory glands is dependent on the resources available to the organism during larval stages.

We also measured the wing length of males as a proxy for their body size. We found a significant effect of larval density treatment on wing length at both ages. Males from low larval density treatment had a bigger wing length than males from high larval density treatment. Moreover, for the data collected from flies of ‘testis size of 4-days old males’ assay, we found a significant effect of selection with MCU flies having a significantly smaller wing length than MB flies. For other assays as well, although the effect of selection was not significant, there was always a trend of MCU flies having smaller wing length than MB flies (Supplement Table S1 and Table S2 and Supplement Fig. S1).

### 5. Discussion

This study aimed to understand the effect of a single generation of larval crowding environment and the evolutionary consequence of adaptation to larval crowding environment on the investment in reproductive organs in males of *Drosophila melanogaster*. To that end, we used populations of *Drosophila melanogaster* adapted to high-density larval environments and their low-density controls. Our results suggest that resources available during developmental stages have an impact on male investment in reproductive tissues but adaptation to larval crowding environment does not lead to the evolution of increased investment in reproductive tissues in males. We measured reproductive investment in males of two ages- one was before their reproductive peak (4-day old adults), and the other age fell within their reproductive peak period (9-day old adults). Our results suggest that at both ages, the effect of selection history and larval density were similar.

In holometabolous insects, the absolute sizes of internal organs are limited by the size of the exoskeleton, and the adults that eclose from larval culture with poor nutrition have smaller body size as compared to adults eclosing from larval cultures having good nutrition (Mirth and Shingleton, 2012; Poças et al., 2020). As expected, we observed the effect of larval density treatment on the absolute size of reproductive tissues, with males from high larval density treatment having a smaller absolute size of reproductive organs. Additionally, in contrast to a pre-



**Figure 1.** Effect of selection and density treatment on the area of testis (mean +/- 95%CI). x-axis represents populations. (a) absolute area of testis of 4 days old males, (b) absolute area of testis of 9 days old males, HD and LD represents high larval density and low larval density, respectively. N = Sample size, P-value of selection × density treatment interaction.

**Table 2**

Summary of three factor mixed model ANOVA with selection and density treatment as fixed factors crossed with random blocks on body size scaled area of testis (a) 4 days old males (b) 9 days old males. Significant terms are marked with “\*” sign.

|                                       | (a)  |     |      |        |      | (b)  |     |      |        |      |
|---------------------------------------|------|-----|------|--------|------|------|-----|------|--------|------|
|                                       | SS   | DF  | MS   | F      | p    | SS   | DF  | MS   | F      | p    |
| Intercept                             | 5.91 | 1   | 5.91 | 1471.5 | 0.01 | 3.54 | 1   | 3.54 | 4652.2 | 0.01 |
| Selection                             | 0.01 | 1   | 0.01 | 2.14   | 0.25 | 0.01 | 1   | 0.01 | 0.14   | 0.74 |
| Density treatment                     | 0.01 | 1   | 0.01 | 2.66   | 0.21 | 0.01 | 1   | 0.01 | 1.54   | 0.31 |
| Block                                 | 0.02 | 3   | 0.01 | 1.51   | 0.37 | 0.01 | 3   | 0.01 | 0.42   | 0.76 |
| Selection × Density treatment         | 0.01 | 1   | 0.01 | 2      | 0.26 | 0.01 | 1   | 0.01 | 0.01   | 0.96 |
| Selection × Block                     | 0.01 | 3   | 0.01 | 2.79   | 0.22 | 0.01 | 3   | 0.01 | 1.81   | 0.33 |
| Density treatment × Block             | 0.01 | 3   | 0.01 | 1.89   | 0.31 | 0.01 | 3   | 0.01 | 5.38   | 0.11 |
| Selection × Density treatment × Block | 0.01 | 3   | 0.01 | 0.72   | 0.55 | 0.01 | 3   | 0.01 | 1.32   | 0.28 |
| Error                                 | 0.25 | 243 | 0.01 | 0      | 0    | 0.05 | 213 | 0.01 | 0      | 0    |

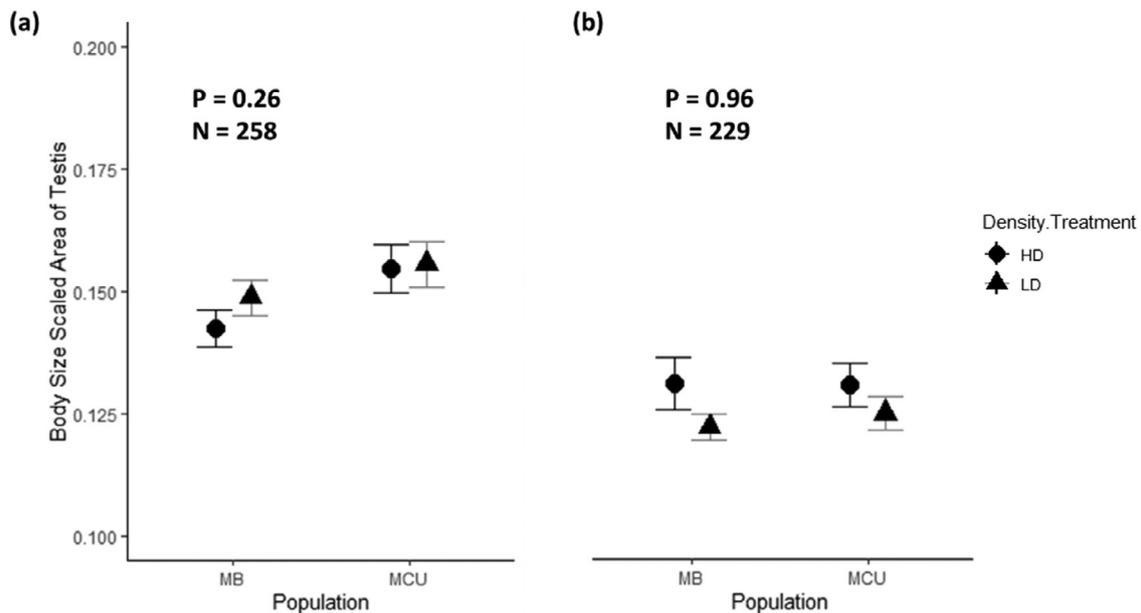
**Table 3**

Summary of three factor mixed model ANOVA with selection and density treatment as fixed factors crossed with random blocks on absolute area of accessory glands (a) 4 days old males (b) 9 days old males. Significant terms are marked with “\*” sign.

|                                       | (a)   |     |       |        |       | (b)   |     |       |        |       |
|---------------------------------------|-------|-----|-------|--------|-------|-------|-----|-------|--------|-------|
|                                       | SS    | DF  | MS    | F      | p     | SS    | DF  | MS    | F      | p     |
| Intercept                             | 36.36 | 1   | 36.36 | 139.24 | 0.01  | 62.36 | 1   | 62.36 | 314.37 | 0.01  |
| Selection                             | 0.13  | 1   | 0.13  | 2.43   | 0.22  | 0.07  | 1   | 0.07  | 2.01   | 0.26  |
| Density treatment                     | 3.28  | 1   | 3.28  | 165.63 | 0.01* | 4.89  | 1   | 4.89  | 110.58 | 0.01* |
| Block                                 | 0.79  | 3   | 0.27  | 5.31   | 0.15  | 0.6   | 3   | 0.2   | 2.93   | 0.15  |
| Selection × Density treatment         | 0.16  | 1   | 0.16  | 7.32   | 0.08  | 0.01  | 1   | 0.01  | 0.07   | 0.82  |
| Selection × Block                     | 0.16  | 3   | 0.06  | 2.42   | 0.25  | 0.1   | 3   | 0.04  | 4.28   | 0.14  |
| Density treatment × Block             | 0.06  | 3   | 0.02  | 0.95   | 0.52  | 0.14  | 3   | 0.05  | 6.12   | 0.09  |
| Selection × Density treatment × Block | 0.07  | 3   | 0.03  | 5.71   | 0.01* | 0.03  | 3   | 0.01  | 1.39   | 0.25  |
| Error                                 | 1.42  | 387 | 0.01  | 0      | 0     | 2.05  | 392 | 0.01  | 0      | 0     |

vious study (Bretman et al., 2016), we observed a negative effect of high larval density treatment on the absolute size of testis and accessory glands size. Bretman et al. (2016) suggest that the positive relationship between larval density and adult body size in their study is because the larvae in their high-density treatment perceive greater risk of competition in their adult stage and invest more in body size and reproductive organs. In our study, the high-density treatment had 600 larvae per 2 ml

of food, while the high-density condition in Bretman et al. (2016) had 200 larvae per 8 ml food. Therefore, in our study, even if larvae would have perceived the high-density as a cue for increased future reproductive competition, there were not enough resources available in the environment to increase their investment in reproductive organs. However, other reasons like the difference in the genetic makeup of the flies used in both the studies could be possible for the observed differences in the



**Figure 2.** Effect of selection and density treatment on testis area (mean  $\pm$  95%CI). x-axis represents populations in all the graphs. a) body size scaled area of testis of 4 days old males, b) body size scaled area of testis of 9 days old males, HD and LD represent high larval density and low larval density, respectively. N = Sample size, P-value of selection  $\times$  density treatment interaction.

**Table 4**

Summary of three factor mixed model ANOVA with selection and density treatment as fixed factors crossed with random blocks on body size scaled area of accessory glands of (a) 4 days old males (b) 9 days old males. Significant terms are marked with ‘\*’ mark.

|   | (a)  |     |      |        |       | (b)  |     |      |        |       |
|---|------|-----|------|--------|-------|------|-----|------|--------|-------|
|   | SS   | DF  | MS   | F      | p     | SS   | DF  | MS   | F      | p     |
| Intercept   | 4.68 | 1   | 4.68 | 267.03 | 0.01  | 8.21 | 1   | 8.21 | 653.19 | 0.01  |
| Selection   | 0.03 | 1   | 0.03 | 4.42   | 0.13  | 0.02 | 1   | 0.02 | 4.78   | 0.12  |
| Density treatment                                   | 0.13 | 1   | 0.13 | 73.66  | 0.01* | 0.11 | 1   | 0.11 | 21.16  | 0.02* |
| Block   | 0.06 | 3   | 0.02 | 2.79   | 0.23  | 0.04 | 3   | 0.02 | 1.52   | 0.32  |
| Selection $\times$ Density treatment                | 0.01 | 1   | 0.01 | 2.89   | 0.19  | 0.01 | 1   | 0.01 | 0.28   | 0.64  |
| Selection $\times$ Block                            | 0.02 | 3   | 0.01 | 3.9    | 0.15  | 0.02 | 3   | 0.01 | 8.81   | 0.06  |
| Density treatment $\times$ Block                    | 0.01 | 3   | 0.01 | 1.05   | 0.49  | 0.02 | 3   | 0.01 | 11.8   | 0.04  |
| Selection $\times$ Density treatment $\times$ Block | 0.01 | 3   | 0.01 | 4.2    | 0.01* | 0.01 | 3   | 0.01 | 0.74   | 0.53  |
| Error   | 0.15 | 387 | 0.01 | 0      | 0     | 0.23 | 392 | 0.01 | 0      | 0     |

results of our study and Bretman et al. 2016. Overall, our results show that there is a negative effect of larval crowding environment on the absolute area of reproductive organs of males from both selected and controlled populations.

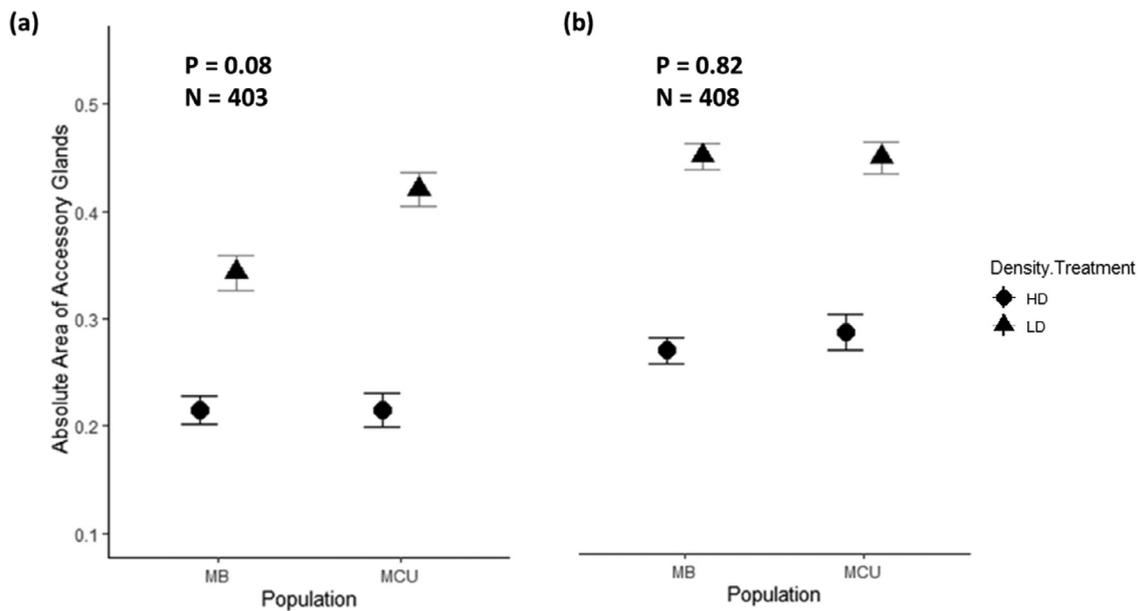
A previous study on the same populations showed that these populations have evolved increased pre-copulatory courtship behavior (Shenoi et al., 2016a). We assessed if selection had favored individuals with increased investment in adult reproductive tissues as well. However, we did not see any effect of selection history on the size of reproductive tissues in these populations. There are at least three non-mutually exclusive reasons that can explain the observed pattern of investment in the larval-crowding adapted populations (MCU) and control populations (MB) populations: (a) our crowding treatment poses a threat to larval survival and therefore promotes increased investment in larval survival traits as opposed to increased investment in adult reproductive tissues, (b) similar mating dynamics across selected and control populations in the adult stages led to similar investments in reproductive tissues in these populations, (c) greater investment in pre-copulatory traits in our crowding adapted populations prevents greater investment in reproductive tissues. These reasons are discussed in detail below:

**a) Investment in larval survival traits may preclude increased investment in reproductive tissues**

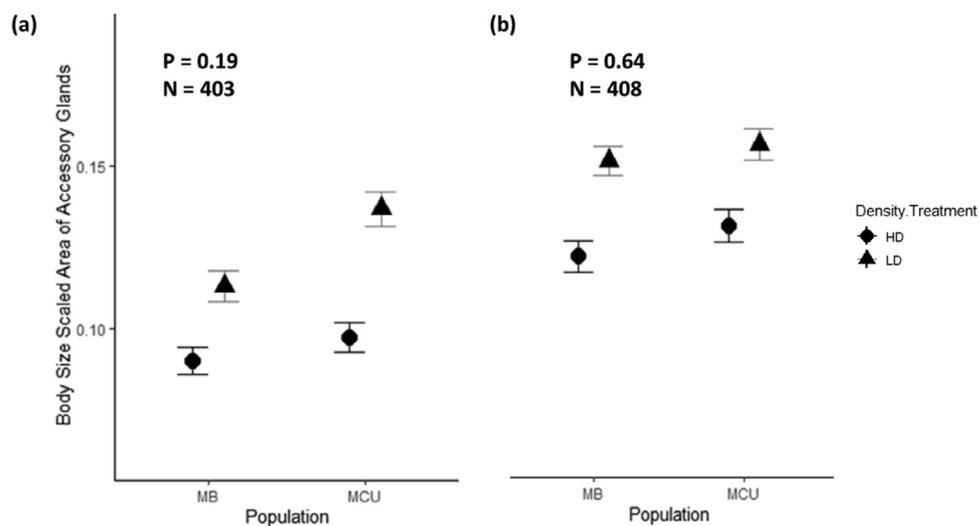
Our crowding-adapted populations go through a larval crowding environment every generation. Only 70-100 larvae out of 800 larvae per vial survive till the adult stage in these populations. Therefore, at severely high larval density, when survival is at stake, individuals with increased investment in larval survival traits might have a fitness advantage. Accordingly, several previous studies on our larval crowding adapted populations and related crowding-adapted populations have shown that larvae of these populations have greater pre-adult survivorship compared to the control populations. They also show a correlated evolution of several survival-related traits such as increased urea tolerance, increased feeding rate, decrease in the amount of minimum food required for pupation, etc (Nagarajan et al., 2016; Sarangi et al., 2016). Therefore, such increased investment in larval survival-related traits may be selected in these populations even if such investments preclude greater investment in adult reproductive tissues. Additionally, extreme larval crowding limits adult body size and may therefore directly limit the evolution of the size of adult reproductive tissues.

**b) Similar mating dynamics across selected and control populations may promote similar investment in reproductive tissues**

Using *D. melanogaster*, Reuter et al (2008) simultaneously varied male mating rate and sperm competition by creating three different operational sex ratios regimes - Equal (1 female to 1 male), intermediate



**Figure 3.** Effect of selection and density treatment on the area of accessory glands (mean  $\pm$  95%CI). x-axis represents populations. (a) absolute area of accessory glands of 4 days old males, (b) absolute area of accessory glands of 9 days old males, HD and LD represent High larval Density and Low larval Density, respectively. N = Sample size, P-value of selection  $\times$  density treatment interaction.



**Figure 4.** Effect of selection and density treatment on the area of accessory glands (mean  $\pm$  95%CI). x-axis represents populations. (a) body size scaled area of accessory glands of 4 days old males, (b) body size scaled area of accessory glands of 9 days old males, HD and LD represents high larval density and low larval density, respectively. N = Sample size, P value of selection  $\times$  density treatment interaction.

female bias (4 female to 1 male) and extreme female bias (10 female to 1 male). Males in the extreme female-bias regime had higher mating rate and were more sperm limited compared to the other two regimes. After 28 generations of evolution, males in the extreme female-bias regime evolved larger testis size compared to the other two regimes (which did not differ amongst themselves). This indicates that male mating rate and sperm limitation can drive the evolution of testis size. In our crowded and uncrowded populations, sex ratios are equal. From previous published (Shenoi et al., 2016) and unpublished results (see supplementary information for details of observation protocols followed for these experiments), we know that the mating frequencies are similar in both MCU and MB populations ( $\sim 1$  mating per female over the span of 36-hour observation period). Therefore, the absence of difference in testis and accessory glands size across both populations can probably be attributed to the absence of difference in mating frequencies in larval crowding

adapted populations and the control populations. Thus, despite of adaptation to extremely different developmental environments, we did not see any difference in investment in reproductive organs between these populations. However, it is important to note that other factors that influence sperm competition like a female's sperm storage capacity and the level of sperm displacement could also have a substantial effect on post-copulatory competition faced by the males of these populations and, therefore, their investment in reproductive tissue. However, at this point we do not have any data about sperm storage or displacement in these populations.

#### c) Greater investment in pre-copulatory behavior may prevent investment in reproductive tissues

Trade-offs between pre- and post-copulatory traits have been reported several times across various insect systems (Katsuki and Lewis, 2015; Simmons and Emlen, 2006; Yamane et al., 2010). It is

argued that since both types of traits require intensive resources, it is beneficial for an organism to invest in either pre- or post-copulatory traits, depending on the mating dynamics. From previous experiments done on the same populations (Shenoi et al., 2016) we know that the courtship activity of crowding-adapted males is 10% higher than the courtship activity of the control populations. Given that larval crowding decreases adult body size and therefore limits available bodily resources, it is possible that increased investment in pre-copulatory traits in the crowding-adapted populations does not allow increased investment in adult reproductive tissues.

Since the sizes of male reproductive organs are directly affected by the body size of the organism, we scaled the absolute sizes of these organs with their body sizes to get an estimate of investment in reproductive tissues irrespective of body sizes. We found that the effect of larval density treatment was observed only in accessory glands of males and not in testes. This result suggests that in both selected and control populations, the proportion of investment of resources in testes does not change with change in larval growth environment, whereas the investment in accessory glands is dependent on the resources available to the organism during their juvenile stages. The observed result could be because of one of two non-mutually exclusive reasons: (a) It is possible that increased investment in accessory glands is important to maintain increased fitness. However, the diverse protein composition of the accessory glands' secretion may require a greater investment of resources to maintain large accessory glands, and due to severe lack of resources in the developmental environment in our study, the males were not able to maintain bigger accessory gland size. This would also suggest that the amount of resources needed to maintain secretions of the accessory glands is probably greater than the amount of resources needed to maintain the secretion of the testis, which makes accessory gland size sensitive to the larval growth environment unlike testis size, (b) Maintaining testis size is relatively more important to fitness than maintaining accessory gland size. However, our data cannot distinguish between these two possibilities.

While our study has uncovered the evolution of adult traits in the crowding adapted populations, it is difficult to dissect out the individual selection pressures that are responsible for their evolution. Indeed, larval crowding is expected to be a fairly complex selection pressure. As larvae grow in a resource-limited larval crowding environment, the nutritional resources in the environment are replaced by the toxic waste generated by the growing larvae. Therefore, as shown in different sets of studies, crowded larval environment can select for larval traits such as increased competitive ability (Mueller et al., 1988; Nagarajan et al., 2016), increased efficiency of conversion of food to biomass (Sarangi et al., 2016), tolerance to metabolic wastes in the environment (Joshi, 1997), reduced food requirement to complete development (Nagarajan et al., 2016) etc. It is possible that these larval traits are genetically correlated with each other and with various adult fitness-related traits. Additionally, it is also known that poor larval growth conditions can directly affect adult fitness related traits (such as body size). Therefore, larval crowding can affect the evolution of adult traits both directly as well as indirectly. However, given the (a) complex nature of the selection in a crowded larval environment, (b) possible genetic correlations between various larval fitness-related traits, and (c) correlations between larval and adult traits, it is indeed difficult to disentangle the individual selection pressures and their consequent trait responses.

Our results add to the body of literature about the factors affecting investment in reproductive tissue. Previous studies suggest that investment in both testis and accessory gland (measured as size) is affected by both developmental environment and the evolutionary history of the organism. In many species increased density during developmental stages has been shown to increase the body size scaled size of the testis (Gage, 1995; Stockley and Seal, 2001) and absolute size of testis (Johnson et al., 2017), and both, body size scaled and absolute size of the accessory gland (Bretman et al., 2016) (but see Liu et al., 2021). This has typically been explained as a result of the larvae perceiving

the future risk of male-male competition and diverting investment into reproductive tissues accordingly. However, our results suggest that the relationship between larval density and adult reproductive tissue size is probably much more complex and can be mediated through larval resource availability. It is possible that the investment in reproductive tissues in response to larval density follows an inverted U-shaped relationship. At the lower end of the larval density scale, when resources are not limiting, with an increase in larval density, resources are diverted towards reproductive tissues. However, at the higher end of the larval density scale, resources become limiting (like in our study). Therefore, at such densities, investment in reproductive tissues is likely to be small. Our results also suggest that relative investment in different kinds of reproductive tissues might be affected by the available resources and the possible influence of that particular tissue on overall fitness.

## 6. Conclusion

While single generation studies suggest that there is the effect of developmental environment on investment in adult reproductive organs, our results show that adaptation to two extremely different developmental environments does not affect the size of male reproductive tissues in populations of *Drosophila melanogaster*. We suggest that the evolution of investment into reproductive tissues as a consequence of adaptation to a crowded larval environment could be governed by a balance between increased investment in larval survival traits, mating dynamics of the system, and the resources available to the developmental stages.

## Declaration of Competing Interest

Authors describe no competing interest.

## Funding

This work was supported by funding from the Indian Institute of Science Education and Research Mohali, India.

## Author's contribution

The study was conceptualized by RK and NGP. It was carried out by RK, NM, SP. Data analysis was done by RK. RK wrote the first draft. NGP and RK drafted and corrected the final draft of manuscript.

## Data availability statement

All data are available at doi:10.5061/dryad.1ns1rn8vt.

## Acknowledgment

The authors of this manuscript would like to thank all the members of EBL, IISER Mohali for their assistance during this study. We sincerely thank Dr. Manjari Jain and her research group (BEL) for graciously allowing us to use their lab facilities throughout the study, without their help this study would not have been possible. The contribution of Mayank Kashyap, Jigisha, and Aaditya Narasimhan for their inputs in manuscript editing is also acknowledged. Additionally, we acknowledge the Ministry of Human Resource Development (MHRD) and the Indian Institute of Science Education and Research Mohali for funding to carry out this study.

## Funding

This work was supported by funding from the Indian Institute of Science Education and Research Mohali, India.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2021.100027.

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